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ORIGINAL ARTICLE

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MicroRNA-21 Expression is regulated by β-catenin/STAT3 Pathway and Promotes Glioma Cell Invasion by Direct Targeting RECK

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Keywords
β-catenin/STAT3 pathway; Glioma; Invasion; MicroRNA-21 (miR-21); Transcriptional regulation.

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SUMMARY

Aims: MicroRNA-21 (miR-21) expression is increased in many types of human malignancy, including glioma. Recent studies report that miR-21 regulates cell invasion by targeting RECK, however, the underlying transcriptional regulation of miR-21 in glioma cells remains elusive. **Results:** Here, we identify a positive correlation between miR-21 expression and pathological grade in glioma tissues. We demonstrate that β-catenin pathway regulates miR-21 expression in human umbilical vein endothelial cell and glioma cells, and that this regulation is signal transducer and activator of transcription 3 (STAT3)-dependent. Further, chromatin immunoprecipitation and luciferase reporter analysis demonstrate that miR-21 is controlled by an upstream promoter containing a conserved STAT3 binding site. Notably, knockdown of miR-21-inhibited cell invasion by increasing RECK expression and decreased tumor growth in a xenograft model. **Conclusion:** These data provide compelling evidence that β-catenin regulation of miR-21 via STAT3 plays a role in glioma cell invasion and proliferation and indicate that STAT3 is a potential therapeutic target for glioma intervention.

Introduction

Glioma is among the most common neural malignancies in adults. Prognosis depends heavily on the histological grade of the tumor, with the poorest survival rates associates with the most malignant phenotypes [1]. Despite advances in radiation and chemotherapy following surgical resection of the tumor, the prognosis of grade IV malignant glioma, termed glioblastoma (GBM), remains poor, with an average survival time of less than 1 year [2,3]. For this reason, a great deal of research has been devoted to identifying the molecular mechanisms that contribute to malignant progression and invasive growth of glioma [4,5].

The major function of the canonical β-catenin pathway is to regulate cell differentiation and proliferation during development through the β-catenin/T-cell factor-mediated activation of target genes [6]. Altered function of components of the canonical β-catenin pathway is associated with cancer, as this complex regulates transcription of multiple genes involved in cellular proliferation, differentiation, invasion and apoptosis, including STAT3, *c-myc*, and *cyclin D* [7,8]. The sustained activation of the β-catenin pathway has been reported in glioma cells [9]. We have confirmed downregulation of β-catenin pathway suppressed glioma cell proliferation and invasion ability in vitro and tumor growth in vivo [10]. Previously, β-catenin pathway was suggested to regulate STAT3 at the mRNA and protein level, suggesting that STAT3 maybe a direct target of β-catenin pathway. Further investigation confirmed that the β-catenin/TCF4 complex directly bound to the TCF4 binding element site of the STAT3 gene promoter [11]. These data confirm that STAT3 is regulated by β-catenin pathway on the transcriptional level.

β-catenin pathway has been found to regulate the expression of microRNAs (miRNAs), which may play a role in β-catenin-induced invasion ability [7]. miRNAs are small, noncoding RNAs consisting of 20–22 nucleotides that participate in the spatiotemporal regulation of messenger RNA and protein synthesis [12,13]. Emerging evidence suggests that miRNAs are involved in many critical biological processes, including development, differentiation, apoptosis, proliferation, and invasion. As such, miRNA have gained attention as novel targets for cancer therapy. Recent studies have showed that the GBM strongly overexpresses a specific miRNA, miR-21 [14,15]. Indeed, previous reports from our laboratory identified a 7-fold increase in miR-21 expression in glioma cell lines compared to normal brain tissue [16]. Further, several reports indicate that control of miR-21 expression at the transcriptional level is regulated by STAT3, in human glioma cells [17] as well as myeloma and prostate cancer cells [18,19]. Accordingly, we examined whether β-catenin pathway regulates miR-21 expression, and the potential role of STAT3 in its regulation.

Therefore, in these studies, we intended to examine whether the β-catenin pathway transcriptionally regulated miR-21 expression in cultured glioma cells in a STAT3-dependant manner. Moreover, we also tested the effects of miR-21 expression on tumor growth and invasion via regulating expression of *RECK* in a human glioma xenograft model. Finally, we analyzed the expression of miR-21 in 93 glioma tissue samples of different grades, correlating the expression of miR-21 with tumor grade.

Materials and Methods

Human Tissue Samples

These studies were approved by the Tianjin Medical University, Tianjin Huanhu Hospital, and Capital Medical University Institutional Review Board for Human Use. Ninety-three freshly resected glioma samples were collected at the Department of Neurosurgery at Tianjin Medical University General Hospital, Tianjin Huanhu Hospital, and Capital Medical University Tiantan Hospital. Tissue and clinical information were obtained as part of an approved study at the university. The neuropathologist reviewed the clinical diagnoses to classify the pathological grade of the samples according to WHO (World Health Organization) categories (2007). Samples included 46 cases of WHO I-II grade tumors (42 cases of astrocytoma and 4 cases of pilocytic astrocytoma), 16 cases of anaplastic astrocytomas (WHO III grade) and 31 cases of glioblastoma (WHO IV grade). Seven normal brain tissue samples were obtained from internal decompression of patients with cerebral injury and temporal lobe resection for epilepsy. A portion of each tissue sample was snap frozen in the liquid nitrogen following resection and stored at −80°C for isolation of RNA, and the remaining portion was fixed with 10% formalin for histopathological examination [10].

MiR-21 Detection by In Situ Hybridization

In situ hybridization detection of miR-21 in glioma samples and xenograft glioma sections was performed as previously described [16]. LNA/DNA oligonucleotides contained locked nucleic acids at eight consecutive centrally located bases (indicated by the underline) and had the following sequences: LNA-miR-21 5′-TCAACATCAGTCT GATAAGCTA-3′.

qRT-PCR Analysis of miR-21 Expression

qRT-PCR analysis of miR-21 expression in glioma samples was performed as previously described [16]. In brief, total RNA from samples was extracted by Trizol (Invitrogen, USA) and subjected to reverse transcription using a first-strand cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's instructions. The quantitative analysis of the change in expression levels was calculated by real-time PCR machine (7500 ABI, USA). For detection of miR-21, the TaqMan MicroRNA assay kit (Applied Biosystems, USA) was used according to the manufacturer's instructions.

miR-21 DNA Copy Number Analyses by RT-PCR

The miR-21 locus was analyzed for homozygous deletion and gene amplification by RT-PCR assay (forward primer: TTTCTTGCCGTTCTGTAAGTG, reverse primer: TG GATATGGATGGTCAGATGAA). The PCR products were separated by electrophoresis in 2% agarose gels, and the ethidium bromide-stained bands were recorded with a gel documentation system (UVP, Upland, CA, USA). Quantitative analysis of the signals obtained for miR-21 locus was performed with Quantity One software (Bio-Rad, USA).

Cell Culture

Human glioma cell lines (U251, LN229 and SNB19) and human umbilical vein endothelial cell (HUVEC) were utilized in this study and cultured as reported previously [20].

Virus Production, Plasmids, and Oligonucleotides

Human mutant β-catenin expression plasmid was kindly provided by Jinquan Cheng (Departments of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA). The mutant β-catenin was inserted into pcDNA3 vector by Kpn1/Apa1 and Pst1/Pst1, respectively. Shuttle vector pShuttle-CMV and adenoviral vector pAdxsi were obtained from Sinogenomax Co., Ltd. (Beijing, China). Mut-β-catenin cDNA was digested from the pcDNA3 backbone and subcloned into pShuttle-CMV, then transferred into pAdxsi adenovirus backbone, cloned in HEK293 and titered to construct the virus vector encoding mutant β-catenin expression (rAd-mut-β-catenin). HUVEC cells grown to 60% confluence were transduced with rAd-mut-β-catenin at a multiplicity of infection (MOI) of 100.

The pMIR-21-Report-Luc reporter plasmid was purchased from Signosis (Signosis, CA, USA). The mature miR-21 binding sequence was subcloned into a firefly luciferase-based reporter construct immediately downstream of Luc coding sequence, using SPE1/HindIII as follow: ACTAGT TCAACATCAGTCTGATAAGCTAAAGCTT. The target site underlined is a sequence perfectly complementary to mature miR-21 that can bind and repress luciferase gene expression.

Consequently, increased luciferase activity represents decreased expression and activity of miR-21. The pSTAT3-TA-luc reporter plasmid was purchased from Beyotime (Jiangsu, China). The human four STAT3 response elements (underlined) were subcloned into the luciferase reporter construct immediately downstream of the Luc coding sequence, using XhoI/BglIII as follow: CTCGAGTGCTTCCCGAACGTTGCTTCCCGAACGTTGCTTCCCGAACGTTGCTTCCGAACTAG. The target site underlined is perfectly complementary to STAT3 and can bind and to induce expression of luciferase gene expression. The pMir-Report-3′RECK reporter containing the wild-type RECK 3′ UTR (RECK wt) or RECK 3′ UTR with mutations in the potential miR-21 binding sites (RECK mut) were kind gifts of Dr. Anna Krichevsky (Department of Neurology, Brigham and Women's Hospital, Harvard Medical School, MA, USA; Ref. 21]. The 2′-*O*-methyl (2′-OMe-) oligonucleotides were chemically synthesized by SBS Genetech (Beijing, China). The 2′-*O*-Methyl oligos were composed entirely of 2′-*O*-methyl bases with the following sequences: scramble sequence 5′-AAGGCAAGCUGACCCUGAAGU-3′, 2′OMe- miR-21 antisense (AS-miR-21) 5′-GUCAACAUCAGUCUGAUAAGCUA-3′ [22]. The target siRNA sequence against β-catenin was 5′-CAGGGGGUUGUGGUUAAGC UCUU-3′. A scramble siRNA sequence (5′-TTCTCCGAACGTGTCACGT-3′) was used as a control (Gima Biol Engineering Inc., Shanghai, China; Ref. 23]. Cells were transfected using Lipofectamine 2000 (Invitrogen), following manufacture's instruction.

Western Blot, Immunohistochemistry, Transwell, and Luciferase Reporter Assay

Western blot, immunohistochemistry, and Transwell assays were performed as previously described [24]. For reporter assays, cells were cultured in 96-well plates and transfected with luciferase reporter plasmid. Following 48 h incubation, luciferase activity was measured using a dual-luciferase reporter system (Promega).

ChIP Assay

HUVEC and U251 cells were cultured to 70% confluence. Cells were harvested for chromatin immunoprecipitation (ChIP) by EZ-ChiP kit (Upstate, USA), according to manufacturer's protocols. In brief, solubilized chromatin was prepared from a total of 2×10^7 cells. The chromatin solution was diluted 10-fold with ChIP dilution buffer and precleared with protein A beads and preimmune serum. The precleared chromatin solution was divided and utilized in immunoprecipitation assays with either anti-STAT3 (Santa Cruz, USA) or anti-IgG antibody. Following washing, the antibody-protein-DNA complex was eluted from the beads. After cross-linking, protein and RNA were removed and the purified DNA was subjected to PCR with primers specific for the miR-21 upstream region containing the STAT3 binding sites, forward: 5′-CCTCTGAGAAGAGGGGACAA-3′, reverse: 5′-ACCGCTTCCAGCAA AAGAGT-3′ [18]. Amplified PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by BioImage.

Subcutaneous Tumor Model and Gene Therapy

The nude mouse bearing subcutaneous LN229 glioblastoma xenograft model was performed as previously described [23]. When tumors reached approximately 5 mm in length, the mice were divided into six groups (6 mice per group) randomly. Two groups of mice were treated with AS-miR-21 (200 pmol oligonucleotides in 10 μL Lipofectamine 2000, local injection, once every 3 days for 21 days) or WP1066 (a selective inhibitor of STAT3 phosphorylation, 40 mg/kg, intraperitoneal injection, every other day until six doses had been given) alone. Mice in another two groups were co-treated with WP1066 and miR-21 mimics or AS-miR-21 and siRNA-RECK, respectively. The tumor volume was measured with a caliper every 3 days, using the formula volume $=$ length$\times$ width$^2/2$.

Statistical Analysis

The results presented are the average of at least three experiments each performed in triplicate with standard errors. Statistical evaluation for data analysis was determined by *t*-test. Differences with $P < 0.05$ were considered statistically significant.

Results

miR-21 Expression Correlates with Glioma Histological Grade

Although miR-21 is known to be aberrantly expressed in malignant glioma [16], we assessed miR-21 expression in 93 primary human glioma tissue specimens of various grade to study the role of miR-21 in glioma tumorigenicity. Using in situ hybridization with a nucleic acid-enhanced miR-21–specific probe, we identify that miR-21 expression increases with increasing grade (Figure 1A). Although staining in grade I–II tissue was sparse, specific staining increased in grade III samples and peaked in grade IV glioblastoma tissue. To confirm the accuracy of in situ hybridization, we examined the expression of miR-21 in each of the 93 samples by qRT-PCR. As shown in Figure 1(B), the results indicated that miR-21 levels remained low in grade I–II glioma samples, and significantly increased in grade III–IV glioma, validating the results observed by in situ hybridization.

To decipher the mechanism underlying miR-21 overexpression in glioblastoma, we investigated aberrations in the gene DNA copy number at the miR-21 locus at 17q23.2 by TaqMan qPCR. Analysis revealed no evidence of homozygous deletion or amplification of the miR-21 locus in any of the 93 glioma samples tested (Figure 1C). These results suggest that regulation of miR-21 expression occurs at the transcriptional or RNase-mediated posttranscriptional processing level, consistent with many miRNAs [25].

β-Catenin Signaling Regulates miR-21 Expression

The β-catenin signaling pathway has been implicated in the regulation of cellular processes such as proliferation, apoptosis, and

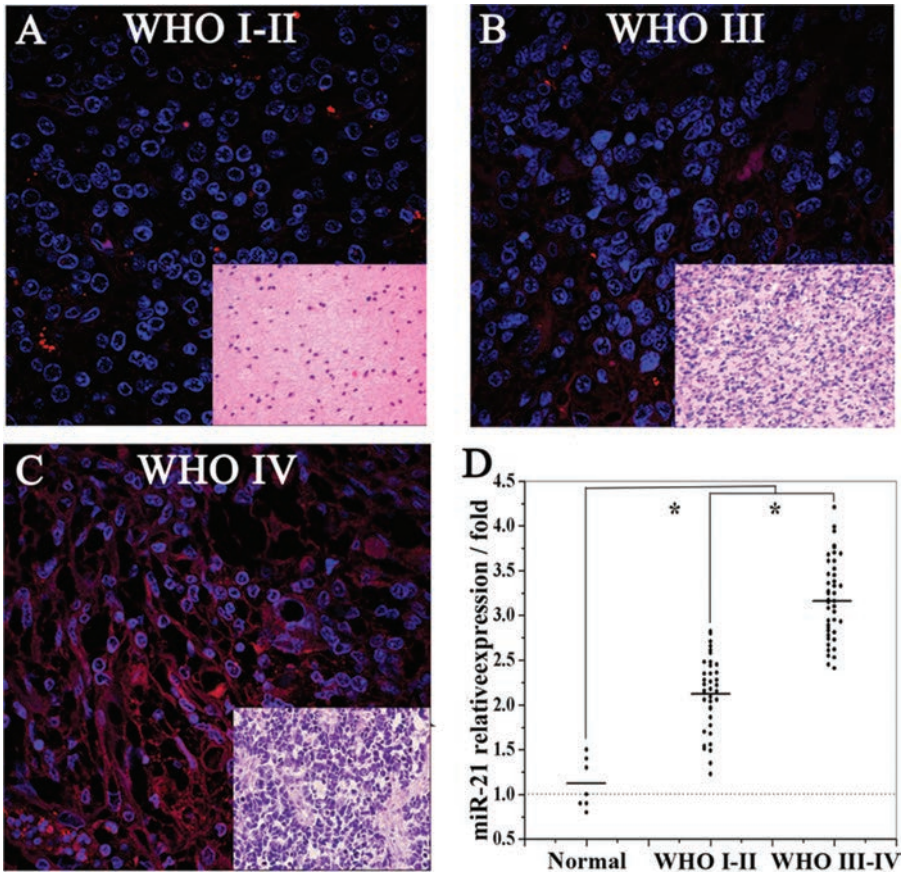

Figure 1 miR-21 expression increases with glioma pathological grade. In situ examination of miR-21 expression and hematoxylin-eosin stain of grade I–II (A), III (B) and IV (C) glioma samples. (D) qRT-PCRs of miR-21 expression, performed with primers specific for mature miR-21, among normal brain tissues (Normal) and glioma samples (grade I–II, grade III–IV). Each reaction was performed in duplicate, and the data is represented as mean ± SEM. $^*P < 0.05$ compared with normal tissue.

cell migration that are similarly impacted by miR-21 expression [16]. To determine whether β-catenin signaling is an upstream regulator of miR-21 expression, we infected HUVEC cells and glioma cells with either mutant constitutively active β-catenin (rAd-mut-β-catenin) or transfected these cells with siRNA specific for β-catenin, and quantified the effects on miR-21 expression by qPCR. As one of the hallmarks of activated β-catenin pathway is the accumulation of cytoplasmic/nuclear β-catenin, the subcellular distribution of β-catenin and miR-21 expression in HUVEC and HUVEC cells treated with rAd-mut-β-catenin were examined by Western blot. Overexpression of β-catenin resulted in cytoplasmic/nuclear accumulation in HUVEC cells. As shown in Figure 2(A), cytoplasmic/nuclear accumulation was accompanied by a significant increase in miR-21 expression, as determined by qPCR ($P < 0.05$). To confirm the role of β-catenin in miR-21 expression, β-catenin was knocked down and the impact on miR-21 expression was quantified. Immunoblot analysis and qRT-PCR of total cell lysates confirmed that cellular expression of β-catenin and a known downstream target gene, *cyclin D1*, were dramatically decreased in LN229 cells transfected with siRNA specific for β-catenin (Figure 2B, top-panel). Similar findings were observed in SNB19 and U251 cells. Knockdown of β-catenin was associated with a significant decrease in miR-21 expression in all three cell lines (Figure 2B, bottom panel). Collectively, these data indicate that β-catenin pathway regulates miR-21 expression in human glioma cells and HUVEC cells.

β-Catenin Regulation of miR-21 Expression is STAT3-Dependant

As β-catenin pathway activates miR-21 expression, and STAT3 directly binds to the miR-21 promoter region in human prostate cancer cells and myeloma cells, we next examined whether β-catenin-induced miR-21 expression is STAT3-dependent. In both glioma and HUVEC cell lines, we investigated the effect of

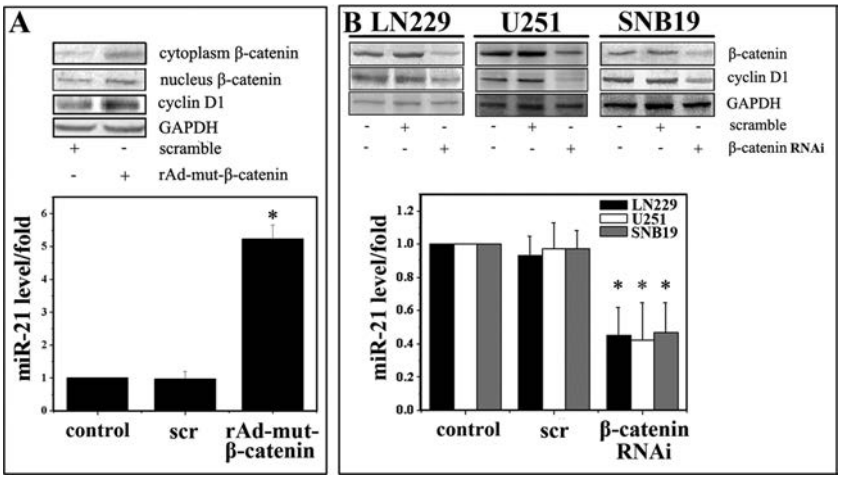

Figure 2 *β*-catenin signaling induces miR-21 expression in human umbilical vein endothelial and glioma cells. (A) Human umbilical vein endothelial cells (HUVEC) were infected with a viral vector encoding mutant *β*-catenin (rAd-mut-*β*-catenin). Protein expression of key components of the *β*-catenin signaling pathway and miR-21 expression were detected by Western blot and qRT-PCR respectively. $^*P < 0.05$ compared to control and scramble groups. (B) LN229, U251, and SNB19 cells were transiently transfected with siRNA targeting *β*-catenin. Protein expression of components of the *β*-catenin signaling pathway and miR-21 expression were detected as in (A). $^*P < 0.05$ compared to control and scramble groups.

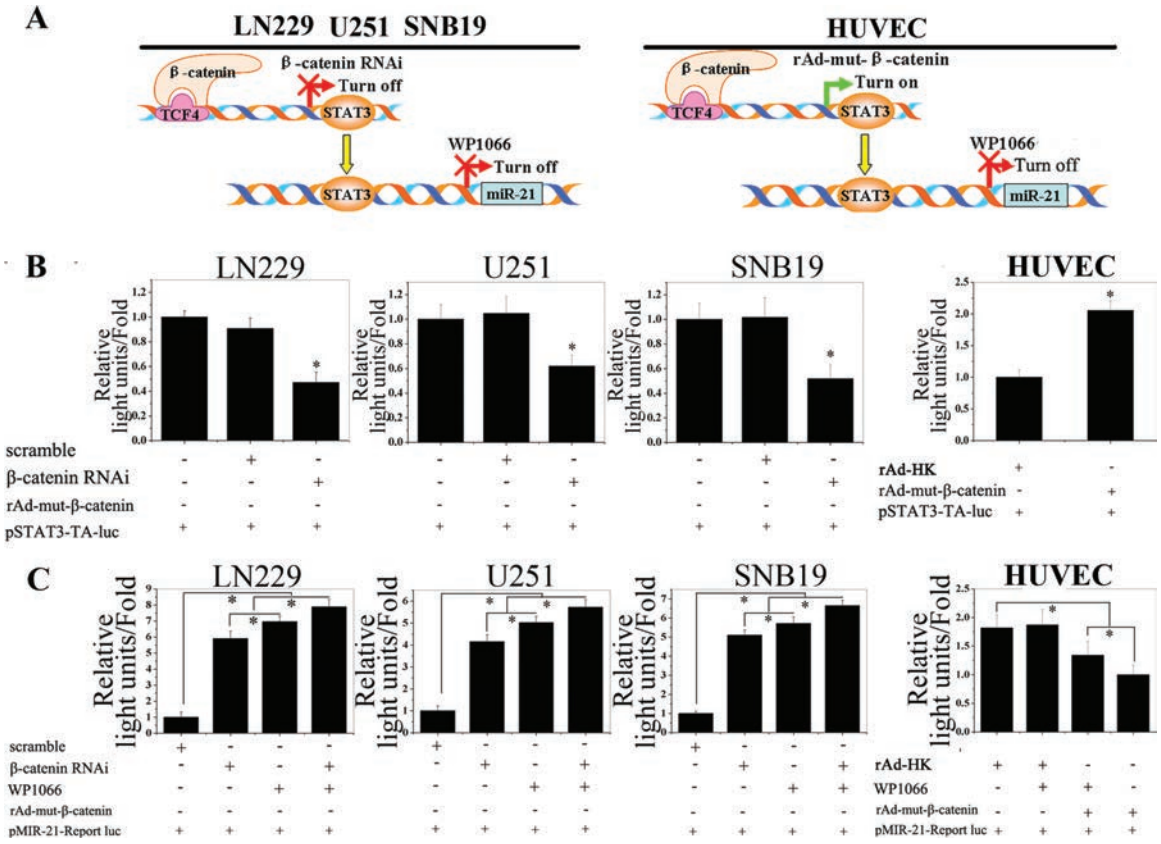

Figure 3 *β*-catenin-mediated regulation of miR-21 expression requires STAT3. (A) A schematic diagram describing the role of *β*-catenin and STAT3 in the transcriptional regulation of miR-21 in LN229, U251, SNB19 and HUVEC cells. (B) Reporter plasmid assays were performed in LN229, U251, and SNB19 cells transfected with either pSTAT3-TA-luc plasmid or siRNA silencing of *β*-catenin expression. Reporter plasmid assays were also performed in HUVEC cells transfected with pSTAT3-TA-luc plasmid or infected with rAd-mut-*β*-catenin. $^*P < 0.05$ compared to scramble groups. (C) Reporter plasmid assays of LN229, U251, and SNB19 cells transfected either with pMIR-21-Report-Luc or with siRNA silencing *β*-catenin expression and treated with WP1066. Reporter plasmid assays were also performed in HUVEC cells transfected with pMIR-21-Report-Luc or infected with rAd-mut-*β*-catenin, and treated with WP1066. Each experiment was repeated eight times. $^*P < 0.05$.

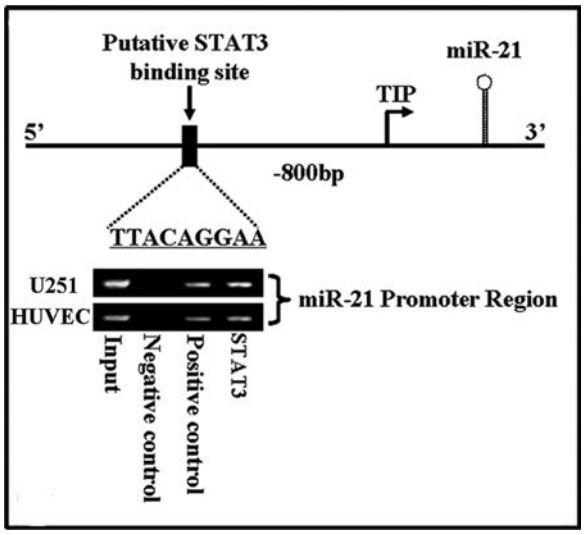

Figure 4 ChIP analysis of STAT3 binding to the miR-21 promoter. U251 and HUVEC cells were subjected to chromatin immunoprecipitation (ChIP) assay, using anti-Stat3. Positive control sample: chromatin immunoprecipitated material obtained with RNA Polymerase II antibody. Negative control sample: chromatin immunoprecipitated material obtained with rabit IgG antibody. Coimmunoprecipitated DNA was amplified by PCR with primers specific for the miR-21 upstream enhancer.

β-catenin signaling on the transcriptional activity of STAT3 by luciferase reporter plasmid (Figure 3A). LN229, U251, SNB19, and HUVEC cells infected with virus encoding β-catenin or transfected with siRNA specific for β-catenin were then transfected with the STAT3 luciferase reporter plasmid pSTAT3-TA-luc. Data indicates that the transcriptional activity of STAT3 is responsive to β-catenin pathway in each cell line, as knockdown of β-catenin significantly reduces luciferase activity while overexpression of β-catenin significantly enhances luciferase activity (Figure 3B). To confirm that β-catenin signaling induced miR-21 via STAT3, the expression of miR-21 was quantified in these cells with or without the addition of the STAT3 inhibitor, WP1066 (10 μM, Sigma). Using the luciferase reporter plasmid, pMIR-21-Report-Luc, the results indicate that when STAT3 activity is inhibited in the presence of WP1066, β-catenin-induced miR-21 expression was significantly reduced (Figure 3C). Taken together, these results demonstrate that STAT3 has an important role in regulating β-catenin-induced miR-21 expression.

As STAT3 is required for β-catenin-mediated miR-21 expression and the putative STAT3 binding site was identified [18], we next examined whether STAT3 directly binds to the miR-21 promoter by chromatin immunoprecipitation (ChIP) analysis. U251 and HUVEC cells were subjected to ChIP using anti-Stat3 or IgG isotype control antibodies. Immunoprecipitated DNA was amplified by PCR with primers specific for the miR-21 upstream enhancer. As shown in Figure 4, STAT3 is indeed recruited to the miR-21 promoter region in both U251 glioma cells and HUVEC cells. These results indicate that STAT3 regulation of miR-21 expression in glioma cells occurs via direct binding to the miR-21 promoter.

miR-21 Regulates Glioma Cell Invasion via RECK

One miRNA usually regulates the mRNA targets in a specific cellular context. Although miR-21 could regulate A172 and LN229 glioma cell invasion by downregulating expression of the MMP inhibitors, TIMP, and RECK [21], we further validate whether miR-21 similarly impact invasive activity of U251 and LN229 cells in vitro and in vivo.

RECK regulates MMP2/9 expression, two matrix metalloproteinases implicated in cell invasion. As miR-21 impacts cell invasion via RECK, we investigated the impact of miR-21 expression on mRNA and protein levels of RECK, MMP2, and MMP9. Western blot and qRT-PCR revealed that RECK protein and mRNA expression was significantly increased upon miR-21 inhibition in glioma U251 and LN229 cells, whereas MMP2/9 protein expression was significantly reduced (Figure 5A–C). To validate that miR-21 directly regulates RECK mRNA transcription at the predicted binding sites, we utilized a luciferase reporter plasmid containing the RECK 3'UTR sequences downstream of firefly luciferase (Figure 5D). LN229 and U251 glioma cells transfected with active or mutant versions of the RECK luciferase plasmid were co-transfected with either siRNA specific to miR-21 or a control scramble oligonucleotide, and luciferase activity was quantified. The results indicate that RECK is a direct miR-21 target (Figure 5E and F).

To measure miR-21 effects on glioma cell invasion, we transfected cells with AS-miR-21 or scramble oligonucleotide and employed a Transwell invasion system. Knockdown of miR-21 expression significantly reduced the quantity of invasive U251 and LN229 cells relative to those transfected with scramble oligonucleotide (Figure 6A). Similarly, in an in vivo subcutaneous LN229 tumor xenograft model, knockdown of miR-21 expression with AS-miR-21 significantly reduced tumor growth compared to scramble groups (Figure 6B, C). Tumors transfected with AS-miR-21 displayed significantly enhanced expression of RECK (Figure 6D). These data indicated that miR-21 had a significant impact on tumor growth and invasion via regulation of RECK.

The Effect of STAT3 and miR-21 on Growth of Glioma Cells in vivo

To investigate the effect of STAT3 and miR-21 on growth of glioma cells in vivo, we further co-treated the LN229 tumor xenograft models with AS-miR-21 and siRNA-RECK or WP1066 and miR-21 mimics (mimic endogenous mature miRNA molecules). Notably, this reduced-growth rate phenotype was significantly rescued by co-transfection of miR-21 mimics with WP1066, indicating that STAT3 regulation of glioma cell growth is mediated, at least partly, by miR-21 (Figure 7). However, this recovered growth was not markedly observed when AS-miR-21 was co-transfected with siRNA-RECK, suggesting that RECK is the major factor that mediates miR-21 function in glioma invasion, not in proliferation (Figure 6B and C). Taken together, these data might conclude that the entire pathway (β-catenin/STAT3/miR-21) is critically involved in those phenotypes of invasion and proliferation of glioma cells.

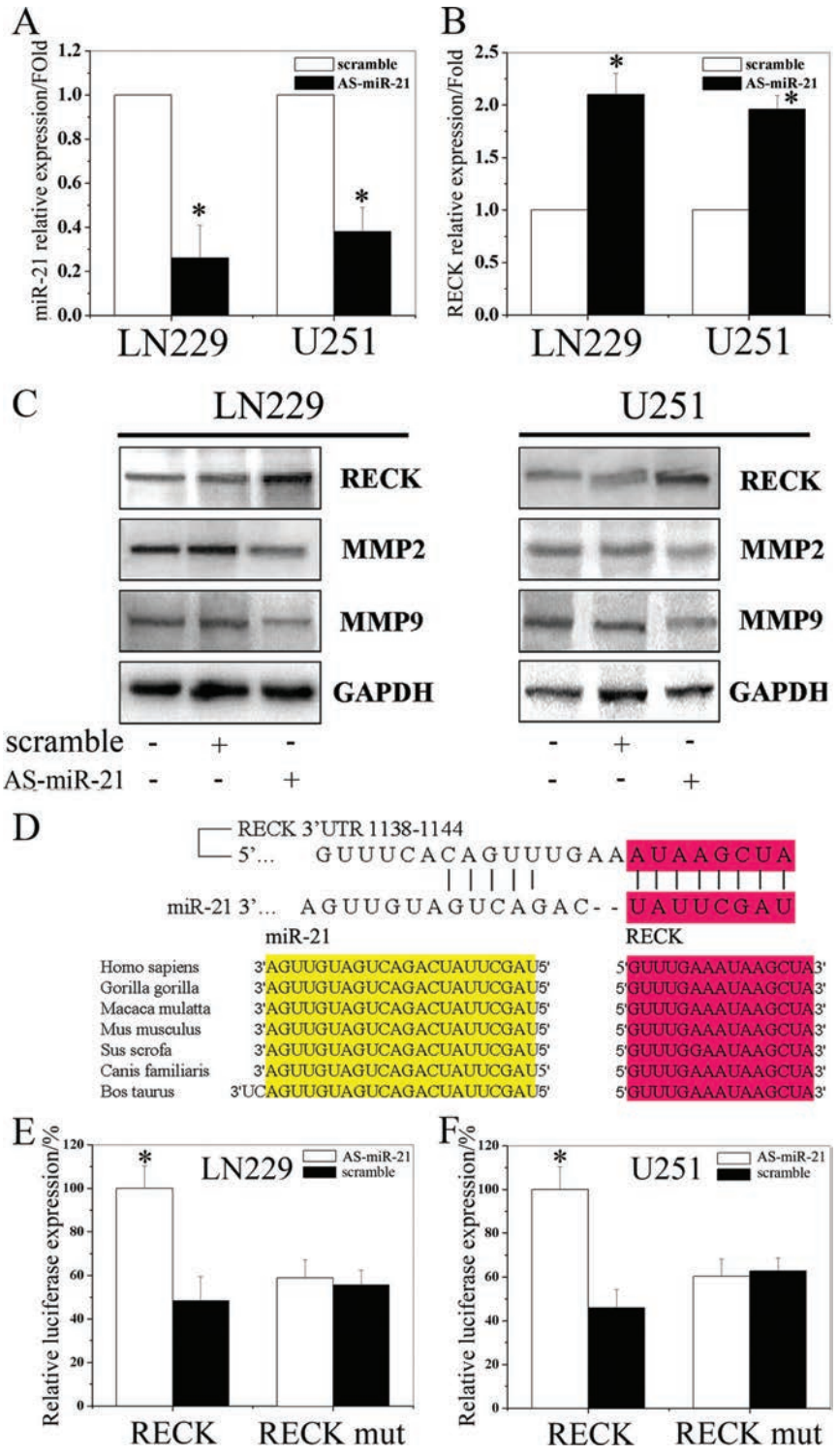

Figure 5 miR-21 targets RECK. (A) Relative expression of miR-21 levels in LN229 and U251 cells transfected with AS-miR-21 for 48 h compared to scramble-treated cells, quantified by qRT-PCR. *$P < 0.05$ compared to control and scramble groups. (B) RECK mRNA level in LN229 and U251 cells transfected with AS-miR-21 for 48 h were quantified by qRT-PCR. *$P < 0.05$ compared to control and scramble groups. (C) RECK, MMP2, and MMP9 proteins level in LN229 and U251 cells transfected with AS-miR-21 for 48 h were detected by Western blot. (D) Predicted miR-21 binding sites within RECK 3'UTRs. Schematic representation of the putative binding sites in RECK mRNAs 3'UTR for miR-21 (identical seed sequences AUCGAAUA as shown). Sequence alignment of miR-21 and the conserved binding sites among the different vertebrate species. (E and F) LN229 and U251 cells were co-transfected with reporter vectors containing the wild-type RECK 3'UTR (RECK wt) or RECK 3'UTR with a mutated miR-21 binding site (RECK mut) and either anti-miR-21 or scramble oligonucleotide. AS-miR-21 transfection resulted in a significant increase in luciferase signal in RECK wt-, but not RECK mut-transfected cells. Reporter experiments were repeated eight times. Data (A–C) represents mean ± SEM from three independent experiments. * $P < 0.05$ compared to scramble group.

Discussion

Dysregulation of β-catenin pathway is observed in many cancers, resulting in aberrant regulation of cell proliferation, metastatic potential, and tumorigenesis [7,8]. Specifically, we have demonstrated that β-catenin pathway regulates glioma cell proliferation and invasion, in part via the AKT pathway [10,26] and GSK-3β [23]. Here, we report that β-catenin pathway could regulate the

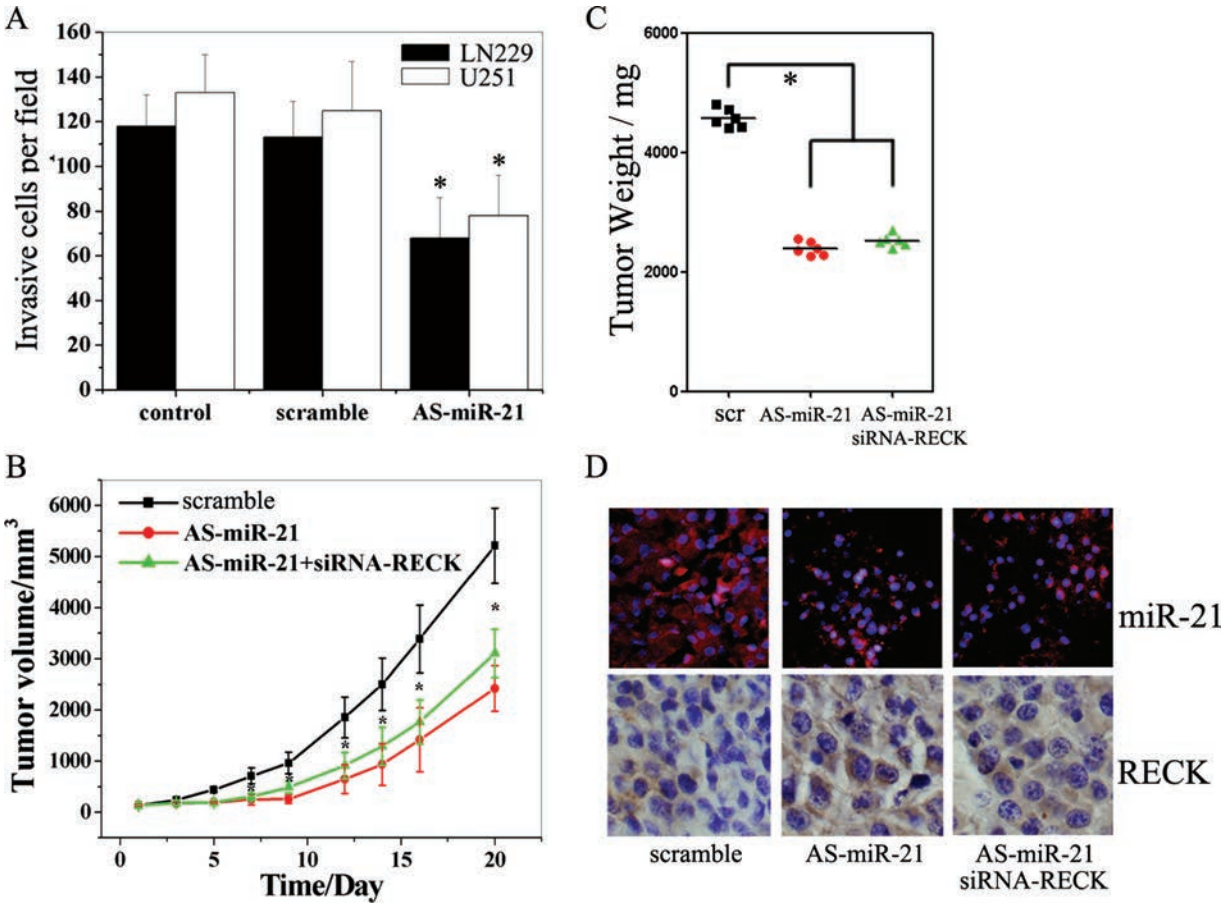

Figure 6 Inhibition of miR-21 reduces glioma growth and invasion in vitro and in vivo. (A) The Matrigel invasion assay was performed on glioma cell lines (LN229 and U251) transfected with either AS-miR-21 or scramble oligonucleotide. Cells that invaded the matrigel membrane were fixed, stained, and observed by optical microscopy. Data represents mean ± SEM of three individual fields from three experiments performed in triplicate for each treatment. $^*P < 0.05$ compared with control and scramble groups. (B) AS-miR-21 or AS-miR-21 and siRNA-RECK were multisite injected into LN229 subcutaneous tumors every 3 days for 21 days. Tumor volumes were measured every 2 days during treatment. $^*P < 0.05$ compared with scramble group. (C) Statistic analysis of tumor weight. $^*P < 0.05$ compared with scramble group. (D) Fluorescence in situ hybridization of miR-21 expression and immunodetection of RECK expression in xenograft tumor sections.

expression of miR-21 (Figure 2). MiR-21 was found to be overexpressed in diverse forms of cancer including glioma, breast cancer, colorectal cancer, stomach and gastric cancer, lung cancer, hepatocellular carcinoma, and prostate cancer [27]. Similar to these findings, we found that increased expression of miR-21 across glioma tissues and cell lines [16]. Moreover, we identify a positive correlation between miR-21 expression and pathological grade in glioma tissues (Figure 1). Reports from our laboratory and others have identified roles for miR-21 in glioma cell apoptosis [28–31], invasion [21], and response to radiotherapy and chemotherapy [32–35], suggesting that miR-21 may be a good target for therapeutic intervention [36–38].

Most importantly, we found that β-catenin pathway could induce miR-21 expression. Several studies have established that miR-21 is induced by various stimuli. For example, Toll ligand receptor activation by LPS upregulates miR-21 in many cells types, including macrophages, fibroblasts, and peripheral blood mononuclear cells [39]. Moreover, IFN-induced miR-21 expression was also dependent on STAT3 and miR-21 plays a critical role in suppressing IFN-induced apoptosis in prostate cancer cells [19]. In this study, we show that miR-21 is induced after β-catenin pathway activation in HUVEC cells, and reduced after β-catenin pathway downregulation in LN229, U251, and SNB19 GBM cells. Thus, miR-21 is a β-catenin pathway target gene in these cells.

On the contrary, STAT3 inhibition significantly attenuated miR-21 expression irrespective of β-catenin, which showed that STAT3 plays an important role in β-catenin-induced miR-21 expression (Figure 3). ChIP analysis on putative STAT3 binding sites in the miR-21 promoter showed that β-catenin induced STAT3 simultaneously bind to miR-21 promoter (Figure 4). This STAT3 binding site was previously identified by ChIP analysis in the upregulation of miR-21 by IL-6 [18]. A recent study reported that miR-21

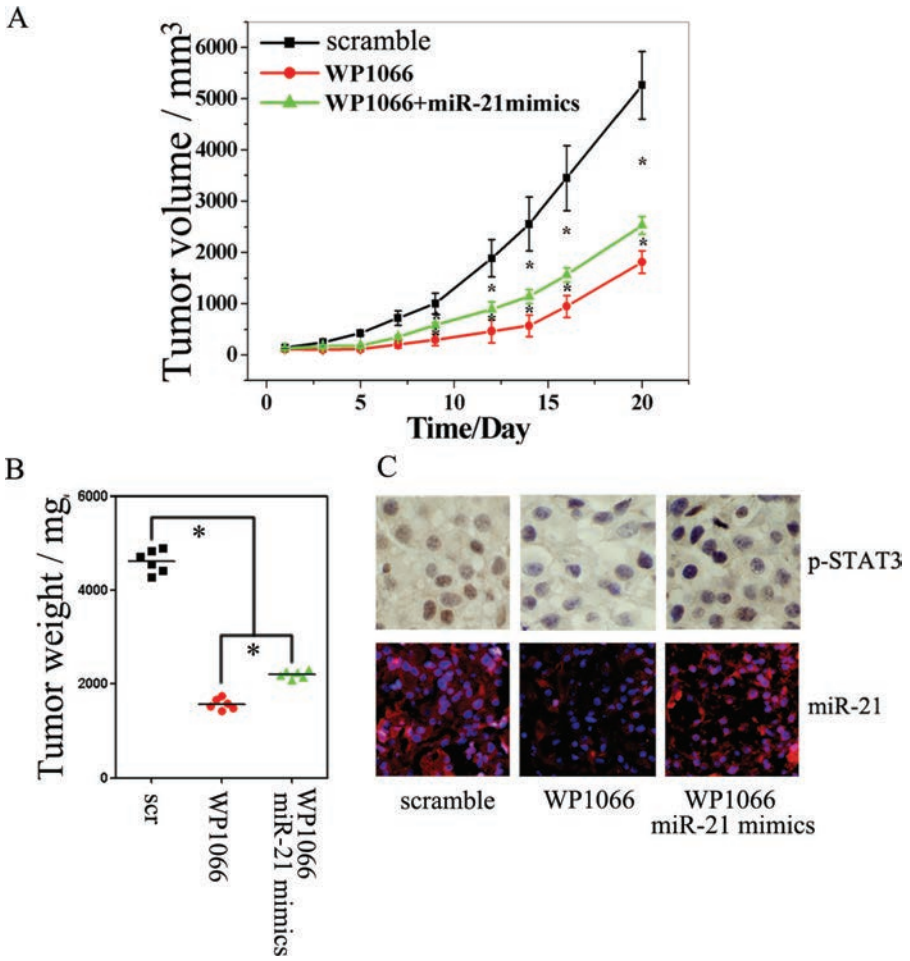

Figure 7 Inhibition of STAT3 reduces glioma growth in vivo. (A) WP1066 or WP1066 and miR-21 mimics were injected into LN229 subcutaneous tumors for 21 days. Tumor volumes were measured every 2 days during treatment. $^*P < 0.05$ compared with scramble group. (B) Statistic analysis of tumor weight. $^*P < 0.05$ compared with scramble group. (C) Fluorescence in situ hybridization of miR-21 expression and immunodetection of p-STAT3 expression in xenograft tumor sections.

expression is negatively regulated by STAT3 activation in human glioma cells, which is in conflict with our findings [17]. The role of STAT3 activation is debatable because its overactivation has been reported to be oncogenic in several cell lines [40,41]. Loffler et al. showed that IL-6–dependent STAT3 activated the transcription of miR-21 in multiple myeloma cells [18]. Yang et al. also confirmed that IFN-induced miR-21 expression was regulated by STAT3 at the level of the miR-21 promoter in prostate cancer cells [19]. The possible explanation of this seemingly paradoxical role of STAT3 activation is that various intracellular and/or environmental cues play a pivotal role in determining the outcome of pathway activation. This discrepancy may arise from the difference in cytokine stimulus and cell type and need to be further confirmed by others [17]. Taken together, these results for the first time show that miR-21 is regulated by the β-catenin/STAT3 pathway.

Further, we have demonstrated in vitro and in vivo, using GBM cells and LN229 glioma xenografts in nude mice, that the entire pathway (β-catenin/STAT3/miR-21) regulates glioma cell proliferation and invasion by directly controlling the miR-21 target, RECK (Figures 5, 6 and 7). RECK is a membrane-anchored MMP inhibitor whose reduced expression or inactivation seems to be critical for the invasiveness and metastasis of various cancers, including glioma cancer. Its expression level is also an important prognostic factor for multiple cancer types [21]. Because glioma tumors and cell lines are heterogeneous genetically, we use the different glioma cell lines from Gabriely's lab to identify the common effects of miR-21 on invasion. These studies further confirmed that STAT3 is the major factor that mediates β-catenin pathway function in glioma proliferation and invasion in vitro and in vivo.

An unresolved question that needs to be addressed is whether β-catenin pathway drives glioma progression. An interesting finding in this work is the direct relationship between miR-21 expression and the pathological grade of glioma samples (Figure 1). The implication is that β-catenin may drive the increase in miR-21

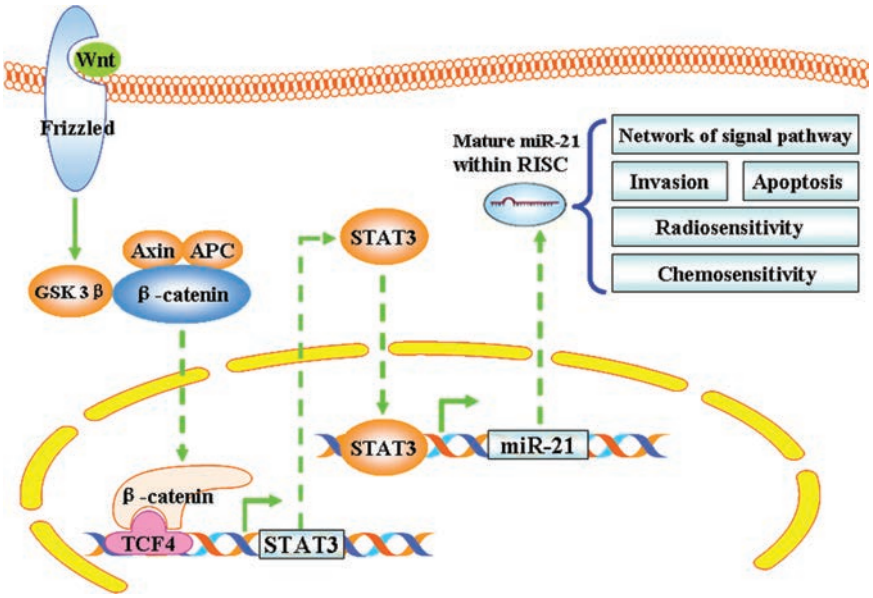

Figure 8 β-catenin-mediated transcriptional regulation of miR-21 via STAT3 and the role of miR-21 on the glioma malignant phenotype. Hypothetical representation of β-catenin-mediated transcriptal regulation of miR-21 via STAT3, and the role of miR-21 in regulation of apoptosis, invasion, chemosensitivity, and radiosensitivity of glioma cells.

expression, thereby resulting progression of the tumor to a higher pathological grade. Alternatively, miR-21 expression may increase during glioma progression independent of β-catenin, however the role of β-catenin in tumor cell invasion strongly supports its role in glioma progression.

In summary, it has been found that the expression of miR-21 was associated with the pathological grade of glioma and the key roles for miR-21 in tumor-related pathways [16,42], apoptosis via PDCD4, caspase 9 and 3 [28–31], invasion via RECK and TIMP [21], chemosensitivity via Bax/Bcl-2 ratio, caspase-3, and LRRFIP1 [32–34], and radiosensitivity [35] in glioma cells (summarized in Figure 8). Our study provides the first evidence that miR-21-mediated β-catenin/STAT3 pathway-induced cell proliferation and invasion via major factor STAT3 in glioma cells. Thus, STAT3 may be a potential prognostic marker and therapeutic target for glioma.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Vredenburgh JJ, Desjardins A, Reardon DA, Friedman HS. Experience with irinotecan for the treatment of malignant glioma. *Neuro Oncol* 2009;**1**:80–91.
2. van den Bent M, Chinot OL, Cairncross JG. Recent developments in the molecular characterization and treatment of oligodendroglial tumors. *Neuro Oncol* 2003;**2**:128–138.
3. Norden AD, Drappatz J, Wen PY. Antiangiogenic therapies for high-grade glioma. *Nat Rev Neurol* 2009;**11**:610–620.
4. Huse JT, Holland EC. Targeting brain cancer: Advances in the molecular pathology of malignant glioma and medulloblastoma. *Nat Rev Cancer* 2010;**5**:319–331.
5. Rao JS. Molecular mechanisms of glioma invasiveness: The role of proteases. *Nat Rev Cancer* 2003;**7**:489–501.
6. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: Components, mechanisms, and diseases. *Dev Cell* 2009;**1**:9–26.
7. Huang K, Zhang J-X, Han L, et al. MicroRNA roles in beta-catenin pathway. *Mol. Cancer* 2010;**9**:252.
8. Dihlmann S, von Knebel Doeberitz M. Wnt/beta-catenin-pathway as a molecular target for future anti-cancer therapeutics. *Int J Cancer* 2005;**4**:515–524.
9. Rossi M, Magnoni L, Miracco C, et al. β-catenin and Gli1 are prognostic markers in glioblastoma. *Cancer Biol Ther* 2011;**8**:753–761.
10. Pu P, Zhang Z, Kang C, et al. Downregulation of Wnt2 and beta-catenin by siRNA suppresses malignant glioma cell growth. *Cancer Gene Ther* 2009;**4**:351–361.
11. Yan S, Zhou C, Zhang W, et al. beta-Catenin/TCF pathway upregulates STAT3 expression in human esophageal squamous cell carcinoma. *Cancer Lett* 2008;**1**:85–97.
12. Kefas B, Comeau L, Erdle N, Montgomery E, Amos S, Purow B. Pyruvate kinase M2 is a target of the tumor-suppressive microRNA-326 and regulates the survival of glioma cells. *Neuro Oncol* 2010;**11**:1102–1112.
13. Lavon I, Zrihan D, Granit A, et al. Gliomas display a microRNA expression profile reminiscent of neural precursor cells. *Neuro Oncol* 2010;**5**:422–433.
14. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 2005;**14**:6029–6033.
15. Rao SA, Santosh V, Somasundaram K. Genome-wide expression profiling identifies deregulated miRNAs in malignant astrocytoma. *Mod Pathol* 2010;**10**:1404–1417.
16. Zhou X, Ren Y, Moore L, et al. Downregulation of miR-21 inhibits EGFR pathway and suppresses the growth of human glioblastoma cells independent of PTEN status. *Lab Invest* 2010;**2**:144–155.
17. Ohno M, Natsume A, Kondo Y, et al. The modulation of microRNAs by type I IFN through the activation of signal transducers and activators of transcription 3 in human

glioma. *Mol Cancer Res* 2009;**12**:2022–2030.
18. Löffler D, Brocke-Heidrich K, Pfeifer G, et al. Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood* 2007;**4**:1330–1333.
19. Yang CH, Yue J, Fan M, Pfeffer LM. IFN induces miR-21 through a signal transducer and activator of transcription 3-dependent pathway as a suppressive negative feedback on IFN-induced apoptosis. *Cancer Res* 2010;**20**:8108–8116.
20. Zhang JX, Han L, Zhang AL, et al. AKT2 expression is associated with glioma malignant progression and required for cell survival and invasion. *Oncol rep* 2010;**24**:65–72.
21. Gabriely G, Wurdinger T, Kesari S, et al. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. *Mol Cell Biol* 2008;**17**:5369–5380.
22. Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: The implications for cancer research. *Nat Rev Cancer* 2010;**6**:389–402.
23. Han L, Yang Y, Yue X, et al. Inactivation of PI3K/AKT signaling inhibits glioma cell growth through modulation of β-catenin-mediated transcription. *Brain Res* 2010;**1366**:9–17.
24. Han L, Zhang A, Wang H, et al. Tat-BMPs-PAMAM conjugates enhance therapeutic effect of small interference RNA on U251 glioma cells in vitro and in vivo. *Hum Gene Ther* 2010, **4**:417–426.
25. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* 2008;**454**:56–61.
26. Yue X, Lan F, Yang W, et al. Interruption of β-catenin suppresses the EGFR pathway by blocking multiple oncogenic targets in human glioma cells. *Brain Res* 2010;**1366**:27–37.
27. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 2004;**2**:281–297.
28. Zhou X, Zhang J, Jia Q, et al. Reduction of miR-21 induces glioma cell apoptosis via activating caspase 9 and 3. *Oncol Rep* 2010;**1**:195–201.
29. Corsten MF, Miranda R, Kasmieh R, Krichevsky AM, Weissleder R, Shah K. MicroRNA-21 knockdown disrupts glioma growth in vivo and displays synergistic cytotoxicity with neural precursor cell delivered S-TRAIL in human gliomas. *Cancer Res* 2007; **19**:8994–9000.
30. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 2005; **14**:6029–6033.
31. Chen Y, Liu W, Chao T, et al. MicroRNA-21 down-regulates the expression of tumor suppressor PDCD4 in human glioblastoma cell T98G. *Cancer Lett* 2008;**2**:197–205.
32. Ren Y, Zhou X, Mei M, et al. MicroRNA-21 inhibitor sensitizes human glioblastoma cells U251 (PTEN-mutant) and LN229 (PTEN-wild type) to taxol. *BMC Cancer* 2010;**10**:27.
33. Shi L, Chen J, Yang J, Pan T, Zhang S, Wang Z. MiR-21 protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase-3 activity. *Brain Res* 2010;**1352**:255–264.
34. Li Y, Li W, Yang Y, et al. MicroRNA-21 targets LRRFIP1 and contributes to VM-26 resistance in glioblastoma multiforme. *Brain Res* 2009, **1286**:13–18.
35. Chaudhry MA, Sachdeva H, Omaruddin RA. Radiation-induced micro-RNA modulation in glioblastoma cells differing in DNA-repair pathways. *DNA Cell Biol* 2010;**9**:553–561.
36. Krichevsky AM, Gabriely G. miR-21: A small multi-faceted RNA. *J Cell Mol Med* 2009;**1**:39–53.
37. Malzkorn B, Wolter M, Liesenberg F, et al. Identification and functional characterization of microRNAs involved in the malignant progression of gliomas. *Brain Pathol* 2010;**3**:539–550.
38. Corsten MF, Miranda R, Kasmieh R, Krichevsky AM, Weissleder R, Shah K. MicroRNA-21 knockdown disrupts glioma growth in vivo and displays synergistic cytotoxicity with neural precursor cell delivered S-TRAIL in human gliomas. *Cancer Res* 2007;**19**:8994–9000.
39. Sheedy FJ, Palsson-McDermott E, Hennessy EJ, et al. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nat Immunol* 2010;**2**:141–147.
40. Bromberg JF, Horvath CM, Besser D, Lathem WW, Darnell JE Jr. Stat3 activation is required 41. Frank DA. STAT3 as a central mediator of neoplastic cellular transformation. *Cancer Lett* 2007;**2**:199–210.
41. Bromberg JF, Horvath CM, Besser D, Lathem WW, Darnell JE Jr. Stat3 activation is required for cellular transformation by v-src. *Mol Cell Biol* 1998;**5**:2553–2558.
42. Papagiannakopoulos T, Shapiro A, Kosik KS. MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. *Cancer Res* 2008;**19**:8164–8172.